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Variations of the Effect of Insulin on Neutrophil Respiratory Burst. The Role of Tyrosine Kinases and Phosphatases

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Abstract—The priming effect of insulin on the fMLP-induced respiratory burst of mouse neutrophils as well as the involvement of tyrosine protein kinases and phosphatases in this process have been studied. Peritoneal evoked neutrophils of NMRI strain mice were incubated with 0.01-100 nM insulin for 1-60 min at 22, 30, or 37°C and activated by 0.1-50 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP). The production of reactive oxygen species (ROS) by neutrophils was monitored by luminol-dependent chemiluminescence. We found that ^{125}I-labeled insulin binding by mouse neutrophils occurred with saturation and high affinity. Insulin itself did not change the basal level of the ROS production but could modulate fMLP-induced respiratory burst. The effect of insulin depended on temperature and duration of pretreatment of the neutrophils with insulin and the concentration combination of the insulin and fMLP. The tyrosine kinase inhibitor tyrphostin 51 decreased the fMLP-induced respiratory burst significantly. Insulin did not change the fMLP response of neutrophils pretreated with tyrphostin. However, the effect of tyrphostin on the response to 50 μM fMLP was considerably decreased in neutrophils treated with insulin. There was no such effect during activation by 5 μM fMLP, for which the priming effect of insulin was not observed. Insulin did not increase the fMLP-induced respiratory burst in neutrophils treated with the protein phosphatase inhibitors orthovanadate and pyrophosphate. If the inhibitors were added after insulin, the combined effect was nearly additive. It is possible that priming by insulin of the fMLP-induced respiratory burst is triggered by tyrosine phosphorylation, realized with its participation, and involves the signaling pathways initiated by tyrosine phosphorylation but subsequently is not dependent on the latter. The role of protein phosphatases in priming by insulin is of little importance. The data indirectly confirm the idea that priming of the neutrophil respiratory burst is a result of crosstalk of signaling pathways of the insulin and fMLP receptors with the participation of tyrosine phosphorylation.

Key words: neutrophil, insulin, respiratory burst, priming, tyrosine kinases, phosphatases, chemiluminescence, radioisotope

Polymorphonuclear neutrophilic granulocytes (neutrophils) provide the fastest defense reactions of an organism against infections. Respiratory burst presents a massive release of reactive oxygen species (ROS) with intensive consumption of oxygen and glucose. It is one of the main cytotoxic functions of neutrophils [1]. Respiratory burst occurs as the result of phosphorylation and assembly of the components of NADPH oxidase. Its intensity is strictly controlled by the regulatory systems of the organism and intracellular signaling systems because excessive ROS production is dangerous for the cells of the host organism [2]. Neutrophils can accept a number of signals and respond to them by different specific reactions. The basis for this flexibility is a diversity of membrane receptors. Data on the insulin receptor of neutrophils are limited and rather contradictory [3, 4]. There are no data on the insulin receptor of neutrophils from an inflammation site and the neutrophils of animals. Some functions of neutrophils can be stimulated by insulin. This establishes the existence of a correlation between specific binding of a hormone [3] and its effects on neutrophil functions [5-7]. Data on the effects of insulin on the oxidase activity of neutrophils are sparse and contradictory [6-9].

In an organism before activation, the neutrophils are usually turned to the "primed" state. In this state,

Abbreviations: ROS) reactive oxygen species; fMLP) N-formyl-methionyl-leucyl-phenylalanine; PKC) protein kinase C; IGF-1) insulin-like growth factor-1; PI3-kinase) phosphatidylinositol 3-kinase; $[Ca^{2+}]_i$) calcium ion concentration in cytosol; MAPKs) mitogen-activated protein kinases; ERKs) extracellular signal-regulated kinases; MEK) kinase of ERKs and MAPKs.

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agents do not activate the cells, but they do prepare them for a faster and stronger response. Such agents include many substances operating through receptors of different types [10, 11]. The mechanism of priming is still under study. It is supposed that amplification of cell activity under priming is a result of additive phosphorylation, which forms the basis of cross-interaction between the signaling systems of the primer and activator [12]. Insulin is not classified as a "primer", in contrast with IGF-1, which has a similar structure [10, 11], though there is reason to believe that insulin may prime neutrophils [7, 13].

The problem of signal transduction during the interaction of insulin with neutrophils remains quite undeveloped. However, there are some works in which this problem is considered. Thus, it was shown that the priming of neutrophil aggregation by insulin could be mediated by activation of MAPKs through p24ras-, Raf-1-dependent pathway [13]. It was found that amplification of neutrophil locomotion by insulin is mediated by activation of a tyrosine kinase associated with a receptor and involving PI3-kinase [5]. The amplification of intracellular generation of peroxide in human neutrophils by insulin was partly explained by activation of PKC [6].

The importance of insulin in regulation of the neutrophil activity is obvious from numerous complications of diabetes mellitus. Their development is more likely to result from the absence of insulin effect than from its influence [14, 15]. However, the mechanism of development of the neutrophil dysfunction in diabetes mellitus remains unexplored. We consider that the reason is that there is no clear knowledge concerning the insulin receptor of neutrophils and its properties and signaling system.

Analysis of the literature revealed that there are a small number of studies of insulin effects on neutrophils. They are rather contradictory, and thus it is impossible to make a clear conclusion about the priming or activating effect of insulin on human neutrophils. In addition, there are very few data on the neutrophils of animals. This situation is probably due to the composite nature of the intracellular signaling network of the insulin receptor, as shown with many cell types [16-18], and the variety of the effects of insulin [19]. The purpose of this work was to define conditions for observing the priming effect of insulin on the neutrophil respiratory burst and investigate the roles of tyrosine protein kinases and phosphatases in this process.

MATERIALS AND METHODS

Materials. Chemicals used in the experiments were as follows: zymosan, Hanks' solution, Hepes, sodium aside, horseradish peroxidase (type VI), chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP), tyrphostin 51, sodium orthovanadate, sodium pyrophosphate, Ficoll (Sigma, USA); bovine insulin (Calbiochem, USA); ^{125}I (Izotop, St. Petersburg, Russia); Urografin (Schering, Germany); dioctyl phthalate, dibutyl phthalate (Merck, Germany); complete medium RPMI 1640 (ICN, USA); 10% fetal bovine serum (Serva, Germany).

Biological material. Peritoneal evoked neutrophils of male mice of outbreed strain NMRI were used in the experiments. The peritoneal cavity was washed out with 3 ml Hanks' solution five hours after intraperitoneal injection of 150 μl of zymosan suspension (5 mg/ml) [20]. The neutrophils were isolated in pure form from the peritoneal cell suspension by centrifugation in Ficoll–Urografin solution of density 1.077 g/ml. Neutrophils comprised nearly 98% of the total number of cells as determined by acridine orange staining. The survival of the cells as estimated by trypan blue staining was more than 95%. The cells were used 1 h after their isolation. Immediately after the isolation, the chemiluminescence (ChL) responses of neutrophils to an activating stimulus were very intensive. This could indicate that the cells were in a primed status just after their isolation. However, because the isolated cells were kept in Ca^{2+}-free Hanks' solution at 4°C, the intensity of ChL gradually decreased and within approximately 1 h it reached a lower level that was stable for about 2 h during an experiment. This behavior of the cells could be due to "depriming" [21]. Before experiments, the cell suspension was placed in experimental mini-dishes with a working volume of 200 μl. The cell density was 10^6 cells/ml.

Measurement of chemiluminescence. The functional activity of the neutrophils was estimated by production of reactive oxygen species (ROS) using the luminol-dependent chemiluminescence technique [22, 23]. For the ChL measurements, we used a ChL-111 chemiluminometer designed in the Laboratory of Nerve Cell Biophysics of the Institute of Cell Biophysics of the Russian Academy of Sciences. Chemiluminescence was measured at 37°C sequentially from 12 mini-dishes; the serial measuring time was 5 sec. The physiological solution for measurements had the following composition: 138 mM NaCl, 6 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM Na_2HPO_4, 10 mM glucose, 10 mM Hepes, 0.135 mM luminol, 0.1 mM NaN_3 and 300 U/ml horseradish peroxidase, pH 7.4. Bovine insulin at 0.01-10 nM was used for priming. The use of recombinant growth factors and insulin is generally considered to require a check for contamination by lipopolysaccharide [8], but since we used native bovine insulin, there was no need for this procedure. Cells were incubated with primer under thermostatted conditions at 22, 30, and 37°C for 20 min. For activation of neutrophil respiratory burst, 1-50 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP) was used. In studies of the combined action of insulin and the inhibitor of tyrosine protein kinases (tyrphostin 51) or inhibitors of protein phosphatases (orthovanadate and pyrophosphate), these chemicals were added to the cell suspension at 1 min intervals.

Assay of ^{125}I-labeled insulin binding. Insulin was iodinated with iodine donor utilizing the ^{125}I isotope as well as with H_2O_2 and lactoperoxidase [24, 25]. The specific activity of the ^{125}I-labeled insulin was $8 \cdot 10^5$ and $1.36 \cdot 10^5$ Ci/mol, respectively. Neutrophil suspension (100 μl) with density $3 \cdot 10^7$ cells/ml in RPMI 1640 medium containing 5% fetal bovine serum and 0.2% sodium azide was incubated with ^{125}I-labeled insulin for 15 min at 37°C or 20 min at 30°C. One thousand-fold excess unlabeled insulin was used for estimation of nonspecific binding. After incubation, 50 μl of the mixture was layered on 350 μl of dioctyl phthalate–dibutyl phthalate (1 : 1 v/v) and centrifuged at 14,000*g* for 3 min [26]. The pellet was counter with a MINI-GAMMA gamma-counter (LKB, Sweden).

Statistical analysis. The data were analyzed as based on the change of the total ROS production during 50 sec from the moment of addition of fMLP as the activator. This approximately corresponds to the change of the maximum of the chemiluminescence intensity (Fig. 1). The total ROS production was calculated as the area under the curve of the time dependence of the chemiluminescence intensity. The effects of insulin and the inhibitors were estimated in percentage as the ratio of the total ROS production of the cells treated with the chemicals to that of untreated cells. The effects are expressed as average value ± mean square error for the indicated number of independent experiments. Student's *t*-criterion was used to ascertain the differences from a control or between the groups, in each case the probability being indicated.

RESULTS

Insulin-induced increase in the neutrophil response to chemotactic peptide. Insulin at 0.01-100 nM concentrations did not change the level of spontaneous chemiluminescence (ChL) of neutrophils, i.e., insulin itself did not initiate the generation of reactive oxygen species (ROS). However, pretreatment of cells with insulin could amplify the response to the chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) at 1-50 μM concentrations (Fig. 1).

Because data on the effect of insulin on the oxidative activity of neutrophils are inconsistent [6-8], to investigate its mechanism it was necessary to determine conditions providing repeatability of experimental results. A potentiating effect of 1 nM insulin on neutrophil respiratory burst induced by 50 μM fMLP was found if the incubation with insulin lasted at least 15 min. The maximal effect was observed within 20 min after addition of insulin; more prolonged incubation resulted in a decreased effect (Fig. 2). Investigation of the priming effect of insulin at different temperatures revealed that 1 nM insulin did not change the responses of neutrophils

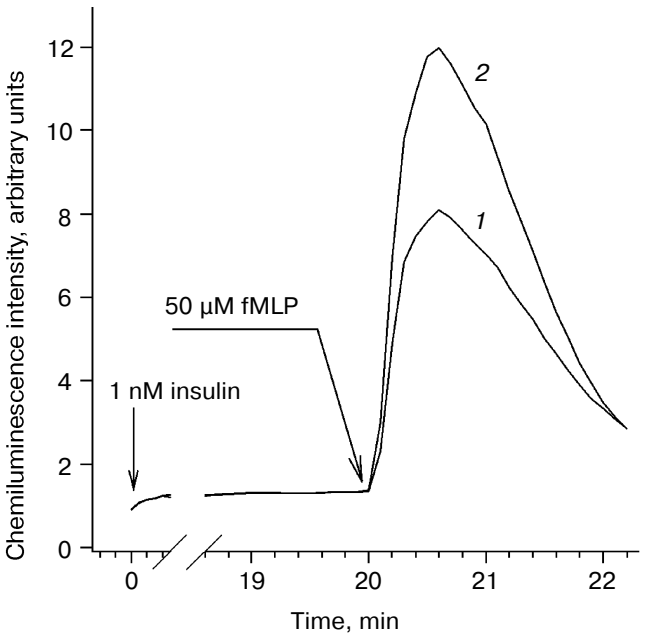

Fig. 1. Kinetic curves of the chemiluminescence response to 50 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP) of control neutrophils (*1*) and neutrophils primed by 1 nM insulin (*2*). The control and primed cells were incubated for 20 min at 37°C and then activated by fMLP. The arrows indicate the moment of addition of primer (insulin) and activator (fMLP).

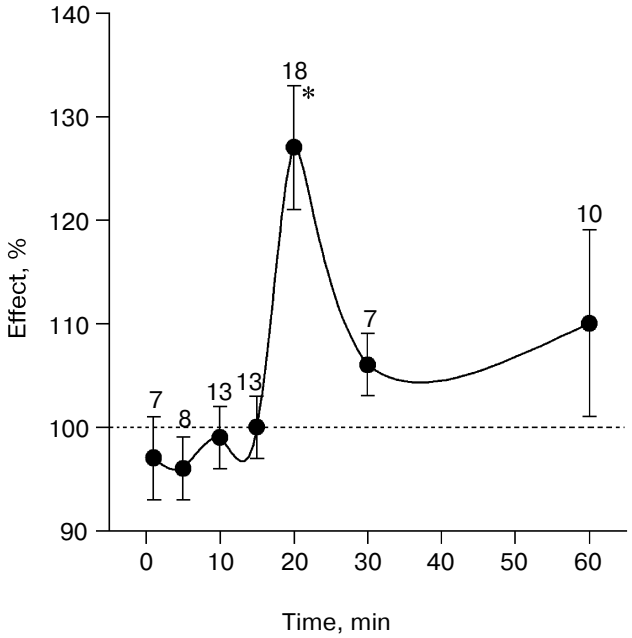

Fig. 2. Time dependence of the priming effect of insulin on fMLP-induced respiratory burst of neutrophils. The cells were treated with 1 nM insulin at 37°C within the time interval indicated on the abscissa, and then the cells were activated by 50 μM fMLP. The average values and mean square errors are indicated. The number of independent experiments is indicated above the experimental points. The response of control cells was taken as 100%; *, significantly different from control, $p < 0.05$.

to 50 μM fMLP at 22 and 30°C, but at 37°C significant amplification of the respiratory burst induced by the same dose of fMLP (28 ± 6%, n = 15) was observed. Conditions for preliminary incubation with insulin were selected based on these experiments: temperature, 37°C; duration, 20 min.

Because the specific binding of insulin by neutrophils and the presence of an insulin receptor on neutrophils remain unclear [3, 4] and there are no data on insulin binding by the mouse neutrophils, we carried out experiments to assess the parameters of binding of this ligand.

Specificity of insulin binding. Investigation of the specificity of insulin binding by mouse neutrophils revealed that the binding of ^{125}I-labeled insulin was of high affinity, and the kinetics of binding showed saturation (Fig. 3a). Analysis of specific binding of ^{125}I-labeled insulin by neutrophils in Scatchard coordinates showed that the cells have two types of binding sites on their surface. The dissociation constants were $K_{d1} = (4.7 \pm 0.4)\cdot 10^{-11}$ M and $K_{d2} = (2.5 \pm 0.7)\cdot 10^{-9}$ M at 37°C (Fig. 3b) and $K_{d1} = (4.8 \pm 0.5)\cdot 10^{-11}$ M and $K_{d2} = (1.8 \pm 0.9)\cdot 10^{-9}$ M at 30°C. The differences between the corresponding constants at 30 and 37°C are uncertain.

The concentration dependences of the priming effect of insulin were studied in the following concentration ranges: 0.01-100 nM insulin; 1-50 μM fMLP. The experiments showed (Fig. 4) that pretreatment of the neutrophils by insulin resulted in a non-monotonic change of the fMLP-responses that depended on the concentrations of insulin and fMLP. At certain combinations of concentrations, the fMLP response of the insulin treated neutrophils did not vary or was slightly decreased. Ranges of insulin concentrations were found where the fMLP responses of the insulin primed neutrophils were amplified: 1) a range of low concentrations of insulin, e.g., combinations of 0.01 nM insulin and 5 μM fMLP (22 ± 10%, n = 3) and 0.1 nM insulin and 1 μM fMLP (36 ± 12%, n = 4); 2) an intermediate range of concentrations of insulin, e.g., the combination of 1 nM insulin and 50 μM fMLP (28 ± 6%, n = 15); 3) a range of high concentrations of insulin, e.g., combinations of 10-100 nM insulin and fMLP in low and high concentrations. It should be noted that the combinations of all used concentrations of insulin with 10 μM fMLP gave no significant effect (Fig. 4, *3*).

Earlier we observed differences in the effect of insulin on neutrophils of mice of the genetically remote strains BALB/c, NMRI, and C57BL. A dependence of the effect of insulin on the conditions of animal housing was detected: the increase of the fMLP response was nearly absent with neutrophils of mice kept in SPF (specific pathogen free) conditions, whereas the increased response was observed for mice adapted to the usual (conventional) conditions [27].

Therefore, there are a number of the factors that influence the character of the reaction of neutrophils to insulin. The basis for these differences might be a diver-

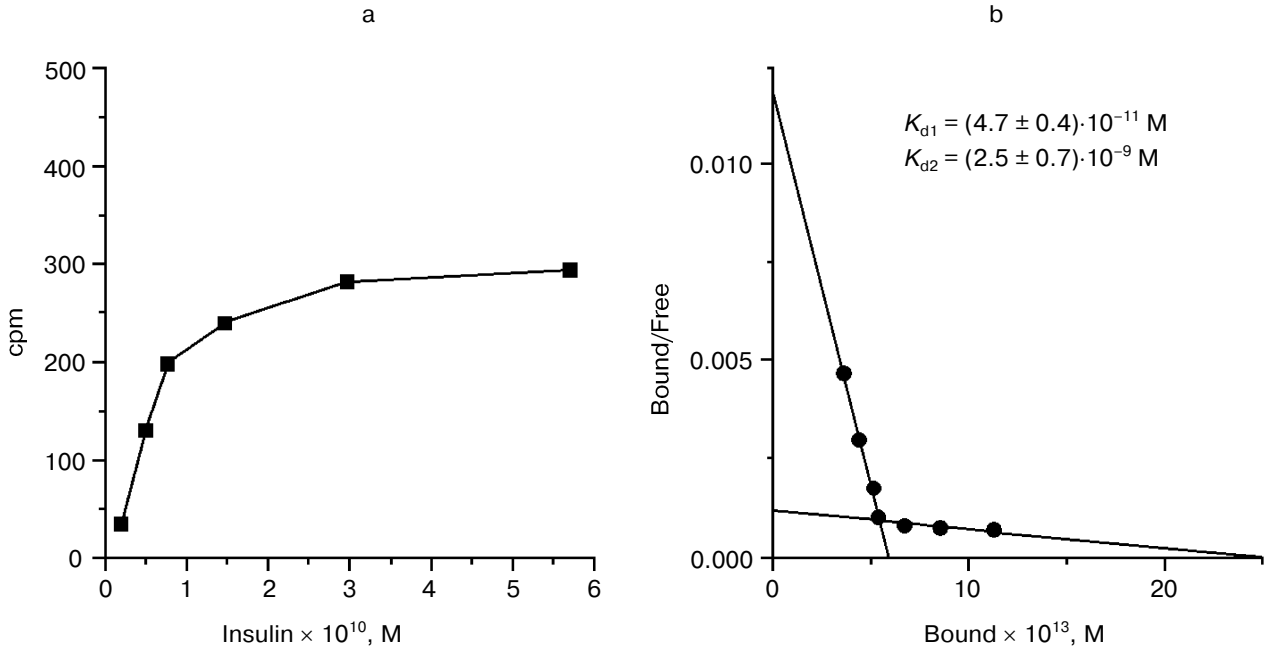

Fig. 3. Specific binding of ^{125}I-labeled insulin with mouse neutrophils (a) and Scatchard plot analysis (b).

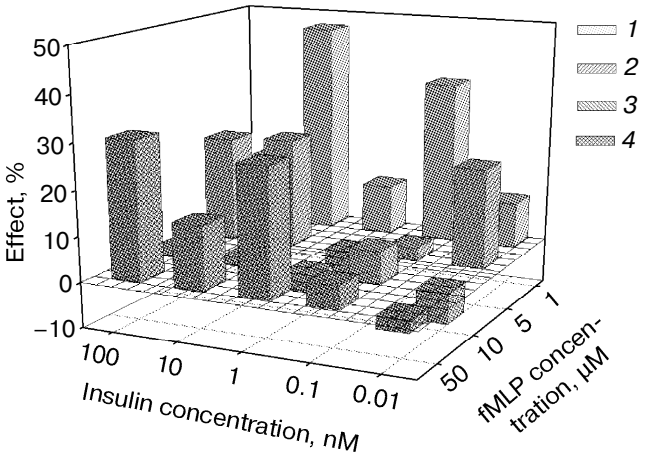

Fig. 4. Modulating effect of insulin on the fMLP-induced respiratory burst of neutrophils is determined by the combination of concentrations of insulin and fMLP used for priming and activation, respectively. Each group of columns from the right to the left shows the change of the insulin effect depending on concentration (0.01-100 nM) on the neutrophil respiratory burst induced by fMLP in concentrations 1 (*1*), 5 (*2*), 10 (*3*), and 50 μM (*4*). The average effects for the studied combinations of concentrations according to the results of 4-15 independent experiments ($N = 109$) are indicated. The concentrations of insulin and fMLP are shown on the horizontal axes. On the vertical axis the effects calculated as the ratio of the difference of the production of ROS by the insulin primed and control cells versus the control cells expressed in percent are shown. Columns extending below the grid show an inhibiting effect of insulin.

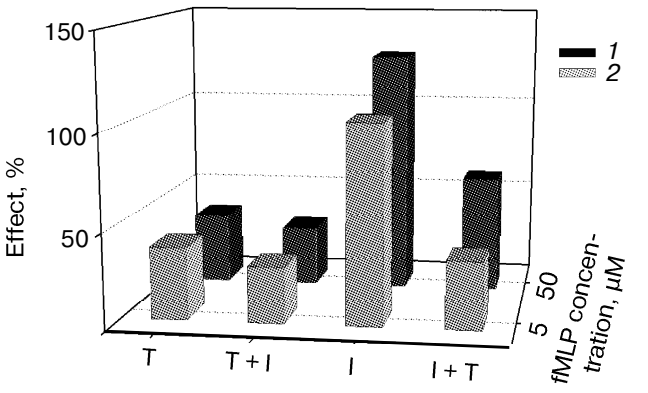

Fig. 5. Effect of tyrphostin 51 on the respiratory burst of neutrophils induced by fMLP at concentrations 5 (*1*) and 50 μM (*2*). Before activation, the cells were treated at 37°C for 20 min with: 5 μM tyrphostin (T); sequentially (with 1-min interval) 5 μM tyrphostin and 1 nM insulin (T + I); 1 nM insulin (I); sequentially (with 1-min interval) 1 nM insulin and 5 μM tyrphostin (I + T). The average values of the effects from 6 independent experiments are shown. The response of control cells was taken as 100%.

gence of signaling pathways of the fMLP receptor originating from the heterogeneity of fMLP binding [28] as well as complexity of the insulin signaling network activated by binding of insulin with its receptor [16-18]. However, as mentioned above, the characteristics of the insulin signaling system in neutrophils have not yet been studied. Studies on the insulin receptor in different cells has shown that binding of the receptor with its ligand initiates autophosphorylation, activation of tyrosine protein kinases, and a subsequent shift of the balance of activities of tyrosine protein kinases and phosphatases [16]. Tyrosine protein kinase is crucial for transduction of the majority, if not all, of the numerous effects of insulin [16, 17].

Role of tyrosine protein kinases. We studied the role of tyrosine protein kinases in the priming by insulin of the fMLP-induced respiratory burst of neutrophils using tyrphostin 51, an inhibitor of tyrosine protein kinases. It is thought that tyrphostins, synthetic inhibitors of tyrosine kinases, are highly effective and selective [29]. They are derivatives of tyrosine and erbstatine, an inhibitor of tyrosine kinases of natural origin that inhibits tyrosine kinase of the epidermal growth factor receptor and cytoplasmic tyrosine kinases belonging to the family of *Src* kinases. It can compete both with substrates and with ATP.

We analyzed the effect of tyrphostin 51 for two fMLP concentrations: 50 μM, at which an increased effect of 1 nM insulin was observed, and 5 μM, at which the effect was lacking (Fig. 5, columns I). In both cases, 5 μM tyrphostin 51 inhibited the fMLP response with the same efficiency (Fig. 5, columns T). This points to the importance of tyrosine phosphorylation in activation of the neutrophil respiratory burst by fMLP. The addition of insulin after 5 μM tyrphostin 51 resulted in slight increase of inhibition of the responses to fMLP at both concentrations (Fig. 5, columns T + I). Therefore, the absence of the effect of insulin in the case of preliminary inhibition of tyrosine kinases confirms the importance of tyrosine kinases in the insulin signaling system in neutrophils. However, in the cells treated with insulin, the effect of tyrphostin 51 was decreased at 50 μM fMLP (Fig. 5, I + T, black column).

We did not detect a similar phenomenon with the activation of neutrophils by 5 μM fMLP, at which the priming effect of insulin was absent (Fig. 5, I + T, gray column). This suggests that different signaling pathways participate in forming the response to fMLP at different concentrations. The priming by insulin of the fMLP-induced respiratory burst is probably triggered by tyrosine phosphorylation and involves the signaling pathways initiated by tyrosine phosphorylation, but is subsequently independent from it. The data are an indirect confirmation of the suggestion of cross-interaction of signaling pathways in priming, but further investigations with more direct measurements are required.

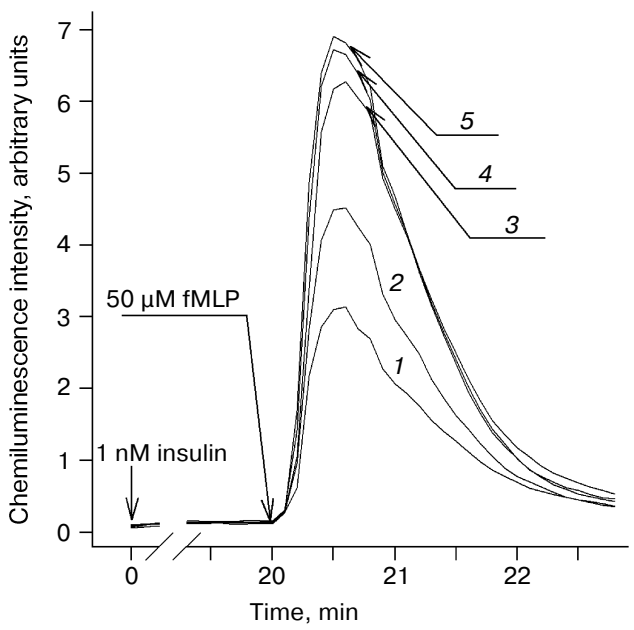

Fig. 6. Effect of orthovanadate, an inhibitor of tyrosine phosphatases, on the respiratory burst of neutrophils. Responses to 50 μM fMLP of control cells (*1*) and cells pretreated at 37°C for 20 min with: 1 nM insulin (*2*); 0.1 mM orthovanadate (*3*); sequentially (with 1-min interval) 0.1 mM orthovanadate and 1 nM insulin (*4*); sequentially (with 1-min interval) 1 nM insulin and 0.1 mM orthovanadate (*5*). The arrows indicate the time of addition of primer (insulin) and activator (fMLP).

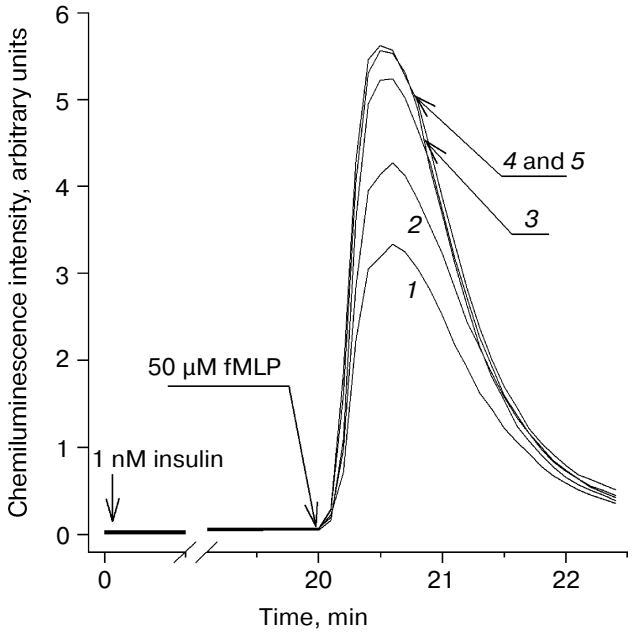

Fig. 7. Effect of pyrophosphate, an inhibitor of phosphatases, on the fMLP-induced respiratory burst. Responses to 50 μM fMLP of control cells (*1*) and cells pretreated at 37°C for 20 min with: 1 nM insulin (*2*); 1 mM pyrophosphate (*3*); sequentially (with 1-min interval) 1 mM pyrophosphate and 1 nM insulin (*4*); sequentially (with 1-min interval) 1 nM insulin and 1 mM pyrophosphate (*5*). The arrows indicate the time of addition of primer (insulin) and activator (fMLP).

Role of protein phosphatases. The role protein phosphatases in the priming of the fMLP-induced respiratory burst of neutrophils was studied using two inhibitors: sodium orthovanadate, a known inhibitor of tyrosine phosphatases [30], and sodium pyrophosphate, a nonselective phosphatase inhibitor [31]. Additional phosphorylation in a cell during priming could result in an increased activity of protein kinases or lowering of the activity of protein phosphatases, which might also be activated by insulin [18].

Pretreatment of neutrophils with 0.1 mM orthovanadate resulted in considerable amplification of the respiratory burst induced by 50 μM fMLP (Fig. 6). The combined effect of 0.1 mM orthovanadate and 1 nM insulin added 1 min later was to increase the respiratory burst by 69 ± 12% (n = 8), which did not reliably differ from the effect of orthovanadate alone, 59 ± 12% (n = 8). The addition of orthovanadate after insulin gave an additive (Fig. 6, curve *5*), though the difference from the effect of orthovanadate only was not reliable.

The effect of pyrophosphate was studied using similar combinations of the substances (Fig. 7). Pyrophosphate (1 mM) strengthened the response to 50 μM fMLP by 62 ± 16% (n = 7). The addition of 1 nM insulin 1 min after pyrophosphate induced an additional increase of the fMLP response, but the difference from the pyrophosphate effect was not reliable, as well as in case of the opposite sequence of addition of the chemical (Fig. 7, curves *4* and *5*). Apparently, protein phosphatases, including tyrosine phosphatases, mainly affect the negative control of the fMLP-induced respiratory burst, but their role in the priming by insulin is minor.

DISCUSSION

Hepatocytes and adipocytes are considered the main targets of insulin in an organism. They are classical objects for study of structure and function of the insulin receptor [16, 17]. These cells have the highest density of insulin receptors (~300,000 receptors/cell). First, the presence of insulin receptors on human circulating granulocytes was shown to bind ^{125}I-labeled insulin [3], but the authors of another work did not find specific binding of insulin by normal human neutrophils [4]. Later, specificity of an effect of insulin on peroxide production by neutrophils was confirmed using monoclonal antibodies [6]. However, data presented in this work are not absolutely convincing because insulin could bind with the

insulin-like growth factor-1 receptor at the concentrations used by the authors [32]. Insulin receptors have not yet been isolated from neutrophils, probably because of their low density (about 1,000 receptors/cell [3]). Our results on binding of ^{125}I-labeled insulin (Fig. 3) have shown that insulin binding by peritoneal mouse neutrophils is of high affinity. The evaluation of two dissociation constants distinguished by almost two orders of magnitude suggests that there are two sites of specific binding of insulin on these cells. We confirmed the presence of two types of insulin binding sites on the membrane fraction of neutrophils. The dissociation constants were $K_{d1} = (1.31 \pm 0.02)\cdot10^{-10}$ M and $K_{d2} = (1.68 \pm 0.01)\cdot10^{-9}$ M at 37°C.

Selecting the optimal conditions for studying of the priming mechanism of insulin on the neutrophil respiratory burst has revealed the dependence of the fMLP-induced response on the duration of incubation with insulin (Fig. 2), temperature, and combination of concentrations of insulin and fMLP (Fig. 4). The data from the literature can be used to make some suppositions. However, additional studies are needed for detailed analysis of the observed dependence.

The temperature effect on the priming by insulin of the fMLP-induced response could result from changes in receptor and post-receptor levels subsequently leading to a change of the NADPH oxidase activity. For example, temperature dependence of the properties of the fMLP receptor [33] and its regulation [34] have been shown. It is possible that the temperature dependence of the parameters of insulin binding noted for human granulocytes [3] is weak under our experimental conditions, as we found no significant difference between insulin binding by mouse neutrophils at 30 and 37°C (Fig. 3). As shown, the intensity of the fMLP-induced response was determined by the initial level of phosphorylation in the cells (Figs. 5-7), and change of temperature could shift the balance of the activities of protein kinases and protein phosphatases, as their activity have marked temperature dependence [35]. The effect of temperature could be due to conformation transitions in the cell membrane resulting in change of activity of membrane-associated enzymes [36].

The time dependence of the priming effect of insulin (Fig. 2) is apparently also due to the parallel involvement of systems of positive and negative regulation of NADPH oxidase. It has been shown that the number of fMLP receptors on the cell surface decreases on prolonged incubation of human neutrophils at 37°C. This was suggested to be due to the internalization of receptors [37]. fMLP induces an increase of cAMP level and activation of protein kinase A in neutrophils [38], and this could depress the activity of NADPH oxidase [2]. Insulin can also influence the cAMP level in cells [39]. It is quite probable that *de novo* protein synthesis contributes to the time dependence of the priming by insulin, as the insulin effect was considerably reduced with cycloheximide, an inhibitor of protein synthesis (data not shown). Besides, long interaction of insulin with neutrophils can result in its inactivation [3, 17]. The differences in the rate of activation of protein kinases and phosphatases might determine the time dependence of the effect of insulin [18]. The character of the time dependence of the insulin effect may be due to differences in the kinetics of activation of the insulin receptor substrates [40].

The dependence of the intensity of the neutrophil response on the combination of concentrations of insulin and fMLP deserves special attention. The non-monotonous character of this dependence (Fig. 4) could be a result of heterogeneity of fMLP binding [29] and insulin binding (Fig. 3). It is known that the responses of neutrophils to fMLP can be divided into two categories: chemotaxis, originating at low concentrations of the peptide, and both secretory degranulation and respiratory burst developing at fMLP concentrations one or two orders of magnitude higher [41]. The effects of insulin in the three concentration ranges mentioned above are apparently mediated by binding of insulin with different parameters: 1) $K_{d1} = (4.7 \pm 0.4)\cdot10^{-11}$ M; 2) $K_{d2} = (2.5 \pm 0.7)\cdot10^{-9}$ M; 3) in addition to high-affinity binding with the insulin receptor, it is possible that insulin binds with a receptor of the insulin-like growth factor-1, whose affinity for insulin is two orders of magnitude lower ($K_d \sim 10^{-7}$ M) [32], this leading to activation of additional signaling pathways.

It is thought that the reason for the diversity of insulin-induced cell reactions is the complexity of the signaling network activated by the binding of insulin with its receptor [16-18]. At least two pathways of signal transduction from the fMLP receptor are known [2, 42, 43]. The effects observed here are probably due to the interaction of signaling pathways of the insulin and fMLP receptors. On varying the conditions of cell priming and activation, interaction at different points may be possible. This supposition is confirmed by the fact that on activation of neutrophils primed with 1 nM insulin, we found no increase of the response to 5 μM fMLP, in contrast to that with 50 μM fMLP (Fig. 5). Here the effect of tyrphostin on cells pretreated with insulin did not vary in response to 5 μM fMLP, possibly because in this case signaling from the insulin and fMLP receptors was realized exclusively by means of tyrosine phosphorylation. Phosphorylation of tyrosine residues in certain enzymes with the priming of oxidase metabolism by various agents has been shown [44-50]. It was found that many agents that activate the NADPH oxidase of neutrophils increased the overall phosphorylation of tyrosine residues in the cells [49-51]. Inhibitors of tyrosine kinases decreased the level of tyrosine phosphorylation in the cells and the superoxide generation induced by fMLP. On the other hand, the inhibition of phosphotyrosine phosphatases caused accumulation of proteins phosphorylated on tyrosine, and, at the same time, increased consumption of oxygen was observed [52].

The kinases causing additional phosphorylation of the NADPH oxidase during the priming have not been identified; MAPKs and ERKs might be involved [10, 12].

In our experiments, tyrphostin significantly inhibited the activation of neutrophils. Insulin did not affect the cells treated with tyrphostin, confirming the role of tyrosine kinases in insulin-related signaling in neutrophils (Fig. 5). However, the effect of tyrphostin was decreased if insulin was added before tyrphostin. This could happen through activation of signaling pathways by insulin required for cell activation by the chemotactic peptide and triggered with the involvement of tyrosine phosphorylation, but subsequently independent from it.

It is difficult to interpret the effects of the phosphatase inhibitors because phosphatases can up- and down-regulate enzymes, in particular those involved in the insulin effect [18]. In our experiments the effect of insulin was not strengthen on addition after the inhibitors of phosphatases orthovanadate and pyrophosphate; on addition of the inhibitors after insulin, the effect of the chemicals was nearly additive (Figs. 6 and 7). We assume that protein phosphatases are of little importance in the priming by insulin of the neutrophil respiratory burst. Their influence probably occurs on a longer time frame (Fig. 2).

It is probable that the effect of orthovanadate on the fMLP-induced respiratory burst of neutrophils is mediated partially by inhibition of ATPases. The influence of orthovanadate on Na^+,K^+-ATPase is well known. However, there are reasons to consider the effects of

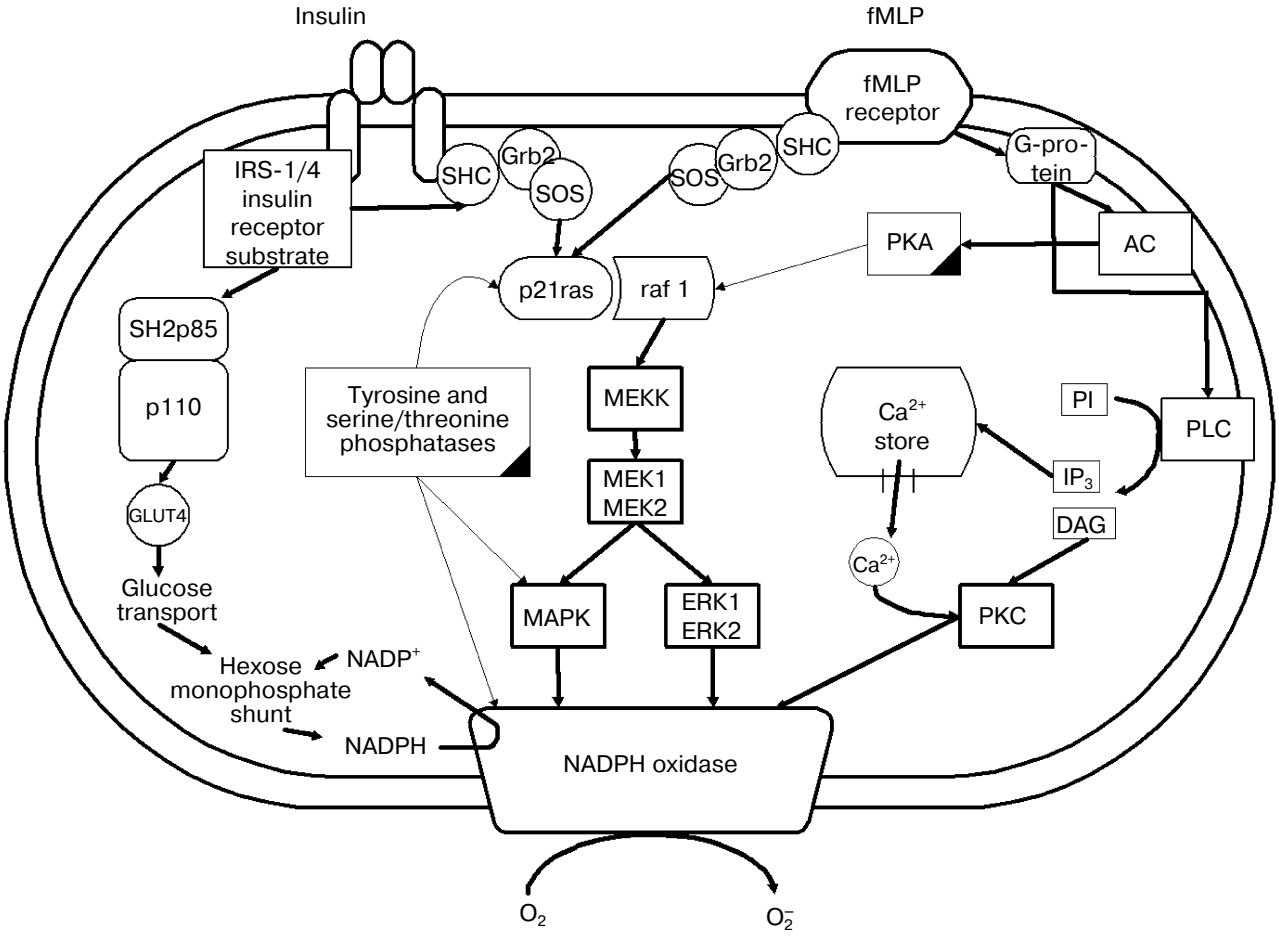

Fig. 8. Some hypothetical and experimentally confirmed mechanisms of regulation of the respiratory burst of neutrophils. Interactions of signaling pathways of the insulin and fMLP receptors may possibly occur at the level of the MAPKs cascade. Abbreviations: fMLP, N-formyl-methionyl-leucyl-phenylalanine; PKA, protein kinase A; AC, adenylate cyclase; PLC, phospholipase C; PKC, protein kinase C; PI, phosphoinositides; IP3, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; IRS, insulin receptor substrate; GLUT4, glucose transporter; MAPK, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; MEK, kinase of ERK and MAPK; MEKK, MEK kinase; SOS, son of sevenless; Grb2, growth factor binding protein 2; p85 and p110, regulatory and catalytic subunits of PI3-kinase, respectively; SH2, SHC, SH-domains of proteins; p21ras, small G-protein; raf 1, protein kinase of p21ras. Denotations: ⟶, upregulation; →, downregulation. The scheme was composed according to [2, 10, 11, 16-18, 41-43, 57].

orthovanadate on the fMLP-induced respiratory burst as being due to the inhibition of tyrosine kinases. The phosphorylation of Na^+,K^+-ATPase on Tyr10 results in its inhibition, and the influence of orthovanadate on Na^+,K^+-ATPase is mediated by inhibition of dephosphorylation of the α-subunit on Tyr10 [53]. According to our data, the effect of orthovanadate is identical in K^+-containing and K^+-free media (28.0 ± 8.9 and 30.5 ± 9.8%, accordingly, $n = 8$). At low concentrations of fMLP, the direction of the effects of orthovanadate and ouabain, a specific inhibitor of Na^+,K^+-ATPase, are opposite (data not shown). It is unlikely that in our experiments orthovanadate acted as a blocker of Ca^{2+}-ATPase since fMLP-induced Ca^{2+} mobilization did not differ significantly in control and insulin primed cells (data not shown). In addition, inhibitors of Ca^{2+}-ATPase induced the generation of ROS [54], which we did not observe for orthovanadate.

A mechanism with a leading role of tyrosine phosphorylation forms the basis for a model of cross interaction of signaling systems in the priming of the neutrophil respiratory burst [10, 12]. In the working scheme (Fig. 8), we have tried to indicate the possible points of cross interaction of signaling pathways of the insulin and fMLP receptors. Binding of insulin with the receptor activates the receptor-tyrosine kinase and subsequent phosphorylation of its substrate. As was shown, phosphatases are also activated [16-18]. There are data indicating that the action of priming agents on cells leads to assembly of complexes of small membrane proteins, which in their incomplete state have no GTPase activity [2]. The activation of these small G-proteins leads to the initiation of the MAPKs cascade and possible additional phosphorylation of components of the NADPH oxidase. This probably causes increased activation of the NADPH oxidase and, therefore, the respiratory burst. Possible points of cross interaction are the components of the NADPH oxidase or MAPKs. Priming could be the outcome of cross interaction between the Ca^{2+}-dependent signaling system operating through serine/threonine kinases, such as PKC or calmodulin-dependent kinases, and signaling systems involving tyrosine kinases: MAPKs, *Src*-kinases and various kinases *Fgr*, *Lyn*, *Fyn*, and *Syk* [55, 56]. It is possible that both systems of phosphorylation are necessary for maximal activity of the NADPH oxidase and that the groups of molecules (or a single molecule) operate as a point of cross interaction between two systems. During activation of the neutrophil respiratory burst, there is phosphorylation of $p47^{phox}$, a component of the NADPH oxidase, on serine residues, which could be a signal for activation of oxidase [57]. Perhaps MAPKs and PKC, causing phosphorylation of components of the NADPH oxidase, form the basis for cross interaction. The activity of MAPKs is regulated and depends on serine/threonine and tyrosine phosphorylation.

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REFERENCES

1. Mayanskii, A. N., and Mayanskii, D. N. (1989) *Sketches about Neutrophil and Macrophage* [in Russian], Nauka, Novosibirsk.
2. Thelen, M., Dewald, B., and Baggiolini, M. (1993) *Physiol. Rev.*, **73**, 797-821.
3. Fussganger, R. D., Kahn, C. R., Roth, J., and De Meyts, P. (1976) *J. Biol. Chem.*, **251**, 2761-2769.
4. Chen, P.-M., Kwan, S.-H., Hwang, T.-S., Chiang, B. N., and Chou, Ch.-K. (1983) *Blood*, **62**, 251-255.
5. Oldenborg, P.-A., and Sehlin, J. (1998) *J. Leukoc. Biol.*, **63**, 203-208.
6. Spagnoli, A., Spadoni, G. L., Sesti, G., Del Principe, D., Germani, D., and Boscherini, B. (1995) *Horm. Res.*, **43**, 286-293.
7. Jin, G. F., Guo, Y. S., Ball, C., and Houston, C. W. (1993) *Regul. Pept.*, **49**, 125-131.
8. Fu, Y.-K., Arkins, S., Wang, B. S., and Kelley, K. W. (1991) *J. Immunol.*, **146**, 1602-1608.
9. Oldenborg, P.-A. (1999) *Inflamm. Res.*, **48**, 404-411.
10. Hallett, M. B., and Lloyds, D. (1997) *The Molecular and Ionic Signaling of Neutrophils*, Landes Bioscience, Austin, Texas, USA.
11. Downey, G. P., Fukushima, T., Fialkow, L., and Waddell, T. K. (1995) *Seminars Cell. Biol.*, **6**, 345-356.
12. Hallett, M. B., and Lloyds, D. (1995) *Immunol. Today*, **16**, 264-268.
13. Pillinger, M. H., Feoktistov, A. S., Capodici, C., Solitar, B., Levy, J., Oei, T. T., and Philips, M. R. (1996) *J. Biol. Chem.*, **271**, 12049-12056.
14. Aleksandrovsky, Ya. A. (1998) *Biochemistry (Moscow)*, **63**, 1249-1257.
15. Shah, S. V., Wallin, J. D., and Eilen, S. D. (1983) *J. Clin. Endocrinol. Metab.*, **57**, 402-409.
16. Cheathman, B., and Kahn, C. R. (1995) *Endocrin. Rev.*, **16**, 117-142.
17. Saltiel, A. R. (1996) *Am. J. Physiol.*, **270**, E375-E385.
18. Virkamaki, A., Ueki, K., and Kahn, C. R. (1999) *J. Clin. Invest.*, **103**, 931-943.
19. Nakipova, O. V., Kokoz, Yu. M., Freidin, A. A., Safronova, V. G., and Lazarev, A. V. (1987) *Fiziol. Zh. SSSR im. I. M. Sechenova*, **73**, 492-498.
20. Van Dyke, K., Van Scott, M. R., and Castranova, V. (1986) *Meth. Enzymol.*, **132**, 498-507.
21. Kitchen, E., Rossi, A. G., Condliffe, A. M., Haslett, C., and Chilvers, E. R. (1996) *Blood*, **88**, 4330-4337.
22. Allen, R. C., and Loose, L. D. (1976) *Biochem. Biophys. Res. Commun.*, **69**, 245-252.
23. Vladimirov, Yu. A., and Sherstnev, M. P. (1989) *Chemiluminescence of Animal Cells*, in *Advances in Science and Technology* [in Russian], Vol. 24, VINITI, Moscow.

24. Salacinski, P. R., McLean, C., Sykes, J. E., Clement-Jones, V. V., and Lowry, P. J. (1981) *Analyt. Biochem.*, **117**, 136-146.
25. Jorgensen, K. H., and Larsen, U. D. (1980) *Diabetologia*, **16**, 546-554.
26. Lundell, D., Lunn, C., Dalgarno, D., Fossetta, J., Greenberg, R., Reim, R., Grace, M., and Narula, S. (1991) *Protein Eng.*, **4**, 335-341.
27. Gabdoulkhakova, A. G., Safronova, V. G., and Sadovnikov, V. B. (2000) *Dokl. RAN*, **372**, 73-76.
28. De Nardin, E., Radel, S. J., and Genco, R. J. (1991) *Biochem. Biophys. Res. Commun.*, **174**, 84-89.
29. Levitzki, A., and Gazit, A. (1995) *Science*, **267**, 1782-1788.
30. Krutetskaya, Z. I., and Lebedev, O. E. (1998) *Roles of Tyrosine Phosphorylation in Regulation of Activity of Ionic Channels of Cell Membranes* [in Russian], Aiyu, St. Petersburg.
31. Cartwright, P. H., Ilchyshyn, A., Ilderton, E., and Yardley, H. J. (1988) *Br. J. Dermatol.*, **118**, 333-338.
32. Gutierrez, J., Parrizas, M., Vaestro, M. A., Navarro, I., and Plisetskaya, E. M. (1995) *J. Endocrinol.*, **146**, 35-44.
33. Hoffman, J. F., Keil, M. L., Riccobene, T. A., Omann, G. M., and Linderman, J. J. (1996) *Biochemistry*, **35**, 13047-13055.
34. Coles, R. B., Ranney, R. R., Freer, R. J., and Carchman, R. A. (1989) *J. Leukoc. Biol.*, **45**, 529-537.
35. Wagerle, L. C., Kim, S. J., and Russo, P. (1996) *Am. J. Physiol.*, **270**, 4645-4650.
36. Wisnieski, B. J., Parkes, J. G., Huang, Y. O., and Fox, C. F. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 4381-4385.
37. Wang, J. M., Hishinuma, A., Oppenheim, J. J., and Matsushima, K. (1993) *Cytokine*, **5**, 264-275.
38. Bengis-Garber, G., and Gruener, N. (1996) *Cell Signal.*, **8**, 291-296.
39. Ortmeyer, H. K. (1997) *J. Basic Clin. Physiol. Pharmacol.*, **8**, 223-235.
40. Inoue, G., Cheatham, B., Emkey, R., and Kahn, C. R. (1998) *J. Biol. Chem.*, **273**, 11548-11555.
41. Snyderman, R., and Pike, M. C. (1984) *Ann. Rev. Immunol.*, **2**, 257-281.
42. Torres, M., and Ye, R. D. (1996) *J. Biol. Chem.*, **271**, 13244-13249.
43. Seger, B., and Krebs, E. G. (1995) *FASEB J.*, **9**, 726-735.
44. Zhang, H., Garlichs, C. D., Mugge, A., and Daniel, W. G. (1998) *J. Physiol. (L)*, **513**, 359-367.
45. Torres, M., Hall, F. L., and O'Neill, K. (1993) *Immunol.*, **150**, 1563-1577.
46. Takemura, O. S., Banno, Y., and Nozawa, Y. (1997) *Biochem. Pharmacol.*, **53**, 1503-1510.
47. Rane, M. J., Carrithers, S. L., Arthur, J. M., Klein, J. B., and McLeish, K. R. (1997) *J. Immunol.*, **159**, 5070-5078.
48. Grinstein, S., and Furuya, W. (1992) *J. Biol. Chem.*, **267**, 18122-18125.
49. Dusi, S., Donini, M., and Rossi, F. (1996) *Biochem. J.*, **314**, 409-412.
50. Downey, G. P., Butler, J. R., Tapper, H., Fialkow, L., Saltiel, A. R., Rubin, B. B., and Grinstein, S. (1998) *J. Immunol.*, **160**, 434-443.
51. Yagisawa, M., Saeki, K., Okuma, E., Kitamura, T., Kitagawa, S., Hirai, H., Yazaki, Y., Takaku, F., and Yuo, A. (1999) *Exp. Hematol.*, **27**, 1063-1076.
52. Li, Y. P., Curley, G., Lopez, M., Chavez, M., Glew, R., Aragon, A., Kumar, H., and Baca, O. G. (1996) *Acta Virol.*, **40**, 263-272.
53. Feraille, E., Carranza, M. L., Gonin, S., Beguin, P., Pedemonte, C., Rousselot, M., Caverzasio, J., Geering, K., Martin, P. V., and Favre, H. (1999) *Mol. Biol. Cell*, **10**, 2847-2859.
54. Nakao, S., Moriya, Y., Furuyama, S., Niederman, R., and Sugiya, H. (1998) *Cell Biol. Int.*, **22**, 331-337.
55. Fernandez, R., and Suchard, S. J. (1998) *J. Immunol.*, **160**, 5154-5162.
56. Yan, S. R., and Novak, M. J. (1999) *FEBS Lett.*, **451**, 33-38.
57. Nauseef, W. M. (1991) *J. Biol. Chem.*, **266**, 5911-5917.